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The association of Duffy binding protein region II polymorphisms and its antigenicity in *Plasmodium vivax* isolates from Thailand

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ABSTRACT

Plasmodium vivax Duffy binding protein II (DBPII) plays an important role in reticulocyte invasion and is a potential vaccine candidate against vivax malaria. However, polymorphisms in DBPII are a challenge for the successful design of a broadly protective vaccine. In this study, the genetic diversity of DBPII among Thai isolates was analyzed from *Plasmodium vivax*-infected blood samples and polymorphism characters were defined with the MEGA4 program. Sequence analysis identified 12 variant residues that are common among Thai DBPII haplotypes with variant residues L333F, L424I, W437R and I503K having the highest frequency. Variant residue D384K occurs in combination with either E385K or K386N/Q. Additionally, variant residue L424I occurs in conjunction with W437R in most Thai DBPII alleles and these variants frequently occur in combination with the I503K variant. The polymorphic patterns of Thai isolates were defined into 9 haplotypes (Thai DBL-1, -2, -3, etc.…). Thai DBL-2, -5, -6 haplotypes are the most common DBPII variants in Thai residents. To study the association of these Thai DBPII polymorphisms with antigenic character, the functional inhibition of anti-DBPII monoclonal antibodies against a panel of Thai DBL variants was characterized by an in vitro erythrocyte binding inhibition assay. The functional inhibition of anti-DBPII monoclonal antibodies 3C9, 2D10 and 2C6 against Thai variants was significantly different, suggesting that polymorphisms of Thai DBPII variants alter the antigenic character of the target epitopes. In contrast, anti-DBPII monoclonal antibody 2H2 inhibited all Thai DBPII variants equally well. Our results suggest that the immune efficacy of a DBPII vaccine will depend on the specificity of the anti-DBPII antibodies induced and that it is preferable to optimize responses to conserved epitopes for broadly neutralizing protection against *P. vivax*.

1. Introduction

Plasmodium vivax is a major cause of malaria worldwide leading to >50% of the disease outside Africa, mainly afflicting Asia and the Americas with approximately 2.5 billion people at risk from vivax malaria [1]. The re-emergence of *P. vivax* in areas where it was considered eradicated, the emergence of drug resistance, and cases of severe and fatal vivax malaria are evidence that it persists as a significant public health problem. Therefore, an important part of control strategy will be the implementation of a vaccine capable of inducing protective immunity against *P. vivax*.

P. vivax Duffy binding protein (PvDBP) is a 140-kDa type 1 integral membrane protein which belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL-EBP) located within the micronemes of *Plasmodium* merozoites [2,3]. The critical erythrocyte binding motif of DBP is in a 330-amino-acid cysteine rich domain referred to as DBP region II (DBPII) or the DBL domain. DBPII binds Duffy antigen/receptor for chemokines (DARC) on red blood cells. The DBP invasion ligand is considered a strong potential vaccine candidate against *P. vivax* infection in part because anti-DBP antibodies inhibit in vitro DBP-erythrocyte binding, reduce merozoite invasion of human erythrocytes and confer protection against blood stage infection [4–8]. Serological responses to DBP and the inhibitory effect of anti-DBP antibodies against DBP-erythrocyte binding increase with a person's age, suggesting that there is a boosting effect due to repeated exposure through recurrent infection [4,19,7]. These data strongly support that DBP can induce a protective immune response during *P. vivax* infection.

However, PvDBPII is highly polymorphic and *dbpII* alleles have a very high ratio of nonsynonymous to synonymous mutation, suggesting a mechanism consistent with high selection pressure driving DBP allelic diversity as a means for immune evasion [9–11]. Analysis of genetic

Abbreviations: DBPII, Duffy binding protein region II; DBL, Duffy binding ligand domain; DBL-TH, Duffy binding ligand Thai haplotypes.

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diversity of *dbpII* alleles among *P. vivax* isolates from different geographical regions, including Brazil, Colombia, South Korea and Papua New Guinea, shows that polymorphic residues are mostly concentrated in the ligand domain and vary by geographic region [12–14]. A study of *dbp* alleles in Papua New Guinea (PNG) found that the substitution rate within region II was 10 times greater than that found within the *dbp* gene overall [9] and that 93% of DBP polymorphisms were within the central segment of DBPII between cysteines 4 and 7 [9]. Polymorphic residues at position 417, 437 and 503 either singly or in combination changed DBP antigenic character, which significantly changed sensitivity to inhibitory antibodies directed against DBPII [15].

Analysis of field parasites shows that some polymorphic residues in DBPII are unique to one population or geographic region, while some variant amino acids, K371E, D384G, E385K, K386N, N417K, L424I, W347R and I503K are common among global vivax isolates [12,13,16,17]. However, only a few individuals produce anti-DBP responses that broadly inhibit against multiple allelic variants [18,19]. Consequently, the polymorphic nature of PvDBPII represents a major impediment to the successful design of a DBPII protective vaccine against diverse *P. vivax* haplotypes. Better understanding of the nature of genetic polymorphisms in DBPII of *P. vivax* isolates from distinct geographic areas, particularly where a large proportion of *P. vivax* infections occur, as well as determining the correlation between DBPII polymorphisms and antigenic character are important for the rational design of a broadly protective vaccine against vivax malaria. In this study we analyzed the genetic polymorphisms of Thai DBPII variants and their effects on antigenic character by using a set of murine monoclonal antibodies.

2. Materials and methods

2.1. Blood samples and DNA preparation

The study was pursued in the malaria endemic areas of Southern Thailand where both major species of malaria, *P. vivax* and *P. falciparu*m, are common. The 44 blood samples used in this study were collected from acute *P. vivax*-infected volunteers at malaria clinics, Chumphon province, which is in the southern peninsular part of Thailand. The areas are malaria endemic with high prevalence of *P. vivax* infection. The confirmation of *P. vivax* infection was performed by microscopic examination of thin and thick Giemsa-stained blood smears. Acute *P. vivax*-infected volunteers who registered at Malarial Clinics were asked for informed consent under the protocol approved by the Ethic Committee on Human Rights Related to Human Experimentation, Mahidol University (MUIRB2012/162.0510). Ten blood spots were collected on filter paper from each consenting *P. vivax* patient for preparation of parasite isolates. Parasite genomic DNA was extracted with a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA).

2.2. Gene amplification and sequencing of PvDBPII

DBPII genes were PCR amplified by polymerase chain reaction (PCR) using specific primers, PvDBPII F: 5′-TTTGGATCCACGATCTCTAGTGCT ATT-3′ and PvDBPII R: 5′-AAACTCGAGTGTCACAACTTCCTGAG 3′. The amplification reaction was performed using the following thermal cycling condition: 94 °C for 90 s, 30 cycles at 94 °C for 15 s, 60 °C for 35 s and 68 °C for 60 s, followed by a 68 °C extension for 2 min. HiFi platinum Taq DNA polymerase was used in all PCR reactions. PCR products were analyzed on 1.2% agarose gel. PCR products were purified and ligated into the T&A cloning vector (Invitrogen, USA). Each ligation mixture was transformed into *Escherichia coli* DH5α competent cells and positive clones were screened. Sequence analysis was performed on two clones for each parasite isolate. Nucleotide sequences were obtained using the dideoxynucleotide chain termination method (Applied Biosytems, Foster City, CA).

2.3. Analysis of PvDBPII gene sequences

The alignment of complete sequences of PVDBPII genes from 44 isolates was analyzed by CLUSTAL and percent similarity was assessed using BioEdit software. Phylogenetic analysis was used to investigate the association of PvDBPII gene with sequences from different geographic regions. The phylogenetic gene tree was constructed using regions common to all available PvDBPII sequences. Sixty PvDBPII gene sequences found in GenBank were compared to Thai PvDBPII isolates including the sequence from reference strain Sal I (DQ156512), the sequences from Vietnam (DQ156518), Indonesia (EU395591), India (FJ491215, FJ491221 and FJ491237), Iran (EU860428 and EU860432–438), South Korea (AAF25483, AAF25486–489 and AAZ81528), Myanmar (JN255576–587), Sri Lanka (GU143917, GU143922, GU143936, GU1439951, GU143953, GU143972, GU143987 and GU144012), Papua New Guinea (AF289482, AF289635, AF289652, AF291096 and AF469530), Brazil (EU812894, EU812906, EU812917, EU812922, EU812927, EU812933, EU812952, EU812933, EU812942 and EU395588) and Colombia (DQ156513, U50576, U50577, U50583, U50584 and U50588).

2.4. pEGFP-DBPII constructs and site-directed mutagenesis

The pEGFP-DBPII construct contains the Sal I DBPII allele cloned into the pEGFP-N1 plasmid with flanking signal sequences from the herpes simplex virus glycoprotein D1 allowing expression of a GFP fusion protein on the surface of transiently transfected COS7 cells [7]. Site-directed mutagenesis was used to create a panel of pEGFP-DBPII constructs with identical polymorphisms to PvDBPII alleles from Thai field isolates using the pEGFP-DBPII-Sal I construct as the parent template. Mutagenesis was performed using the Stratagene Quickchange mutagenesis kit (Stratagene, La Jolla, CA) as previously described [7,15,20]. The recombinant plasmid DNA was purified using an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, CA).

2.5. Evaluating the inhibitory efficiency of anti-DBPII neutralizing antibodies against Thai PvDBPII haplotypes

We used the COS-7 cell erythrocyte assay to evaluate the ability of previously described monoclonal antibodies to inhibit binding of Thai DBPII and reference Sal I haplotype human erythrocytes. Our previous study describes the production and inhibitory efficiency of monoclonal antibodies 3C9, 2C6, 2D10 and 2H2 raised against the DBL7.18 haplotype (DBL variant allele; accession number AAL79051.1). These antibodies strongly inhibit binding of DBL7.18 to erythrocytes [29]. In this study, monoclonal antibodies 3C9, 2C6, 2D10 and 2H2 were tested against Thai DBPII and reference Sal I haplotypes. The construct used to express Thai DBPII or reference Sal I haplotype on the surface of transiently transfected COS has been used extensively as described [7]. This targeted expression efficiently presents the parasite ligand on the surface of the COS cells and expression was confirmed by detection of the C-terminal GFP tag using epifluorescence.

To evaluate the ability of monoclonal antibodies to inhibit binding of erythrocytes to DBP expressed on the surface of transfected COS7 cells, various concentrations of monoclonal antibodies were preincubated with the transfected cells for 1 h at 37 °C before addition of a 10% suspension of human erythrocytes in each well and then incubated at room temperature for 2 h. Percent binding-inhibition of each monoclonal antibody was determined by assessing the number of rosettes in wells of transfected COS7 cells in the presence of monoclonal antibodies relative to rosettes in wells of transfected cells in presence of medium control. The differences in inhibitory efficiency of each monoclonal antibody to the different Thai DBPII alleles were compared by one-way analysis of variance (ANOVA)

using SPSS software. Experiments for each monoclonal antibody were done in triplicate wells and were repeated two times.

$$\%\,\text{inhibition} = [1 - (\text{number of rosettes in the presence of monoclonal antibodies} / \text{number of rosettes in the presence of medium control})] \times 100.$$

3. Results

3.1. Genetic polymorphism of PvDBPII among Thai isolates

The analysis and comparison of amino acid sequences of Thai PvDBPII against the reference Sal I strain (P22290.2) showed 12 polymorphic residues in Thai vivax isolates (Fig. 1A). The sequence analysis classified the mutation pattern into 9 different haplotypes (DBL-TH1, -TH2, -TH3, -TH4, -TH5, -TH6, -TH7, -TH8 and -TH9). Most amino acid polymorphisms were dimorphic. Only one position K386Q/N showed trimorphic polymorphism. All variant residues described in this study have been reported in other *P. vivax* areas but the pattern of polymorphism was novel. Haplotype DBL-TH1 was the predominant haplotype among Thai vivax isolates (26.3%). The highest frequencies (>50%) of variant DBL-TH amino acids as compared to the reference Sal I sequence were found at positions L333F (78.9%), L424I (73.7%) W437R (73.4%) and I503K (63.2%) (Fig. 1B). Variant L424I occurred in conjunction with W437R in most Thai DBPII alleles and these variants frequently occurred in combination with variant I503K. The comparison of DBL-TH haplotypes with the previously identified PvDBPII sequences revealed that haplotypes DBL-TH2, -TH3, -TH4, -TH5, -TH6, -TH7 and haplotype DBL-TH9 were novel in Thai isolates. Only the DBL-TH1 haplotype was common between Thai and Myanmar isolates.

3.2. Phylogenetic analysis of PvDBPII

Phylogenetic trees were constructed from aligned nucleotide sequences based on the neighbor-joining method using Tamura's three-parameter distance (Fig. 2). The Sal I haplotype served as the reference for each alignment and for constructing phylogenetic trees. The trees showed that the 9 DBL-TH haplotypes were widely distributed among different *P. vivax* isolates from distinct geographic regions (Fig. 2). PvDBPII DNA sequences of Thai isolates were related to isolates from Myanmar whereas they differed from Vietnam, Indonesia, India, South Korea, Colombia, Iran, Sir Lanka, Brazil and PNG isolates. The Sal I reference strain was closely related to Mynmar, Iran, Brazil and Sri Lanka isolates. However, the bootstrap analysis showed that Thai isolates were distinct from the Sal I reference strain. These results indicated that there were common DBPII haplotypes circulating in *P. vivax* endemic areas of Thailand and Myanmar.

A

Reference Name	Amino acid position →	308	333	371	384	385	386	390	417	424	437	475	503
Sal I	Sal I	R	L	K	D	E	K	R	N	L	W	P	I
DBL-7.18	AAL79051.1	S			G		Q		K	I	R		K
DBL-TH1	Thailand Haplotype 1	R	F							I	R	A	K
DBL-TH2	Thailand Haplotype2	R	F	E	G	K	Q		K	I	R		
DBL-TH3	Thailand Haplotype3	R	F		G			H					K
DBL-TH4	Thailand Haplotype4	R	F		G	K	Q	H	K	I	R		K
DBL-TH5	Thailand Haplotype5	R		E	G		N		K	I	R		K
DBL-TH6	Thailand Haplotype6	R			G		H						
DBL-TH7	Thailand Haplotype7	R			G								
DBL-TH8	Thailand Haplotype8	R	F										
DBL-TH9	Thailand Haplotype9	R	F							I	R		K

B

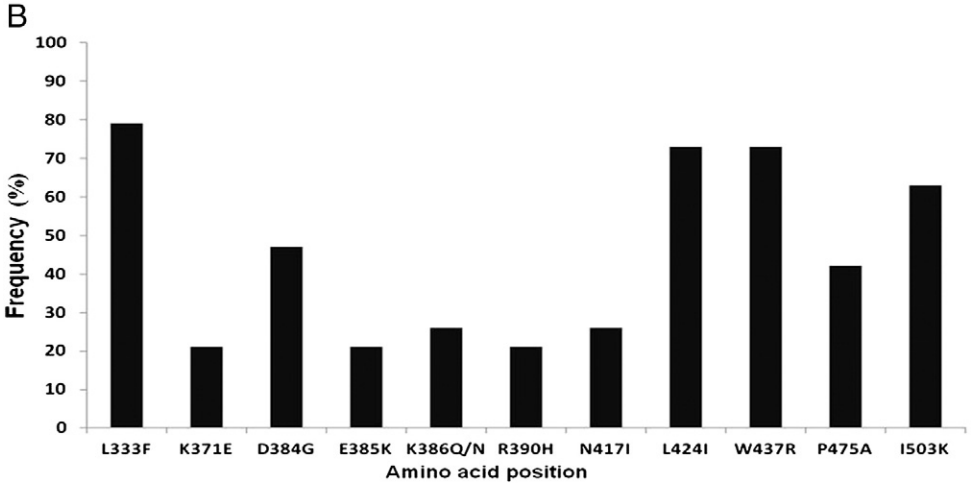
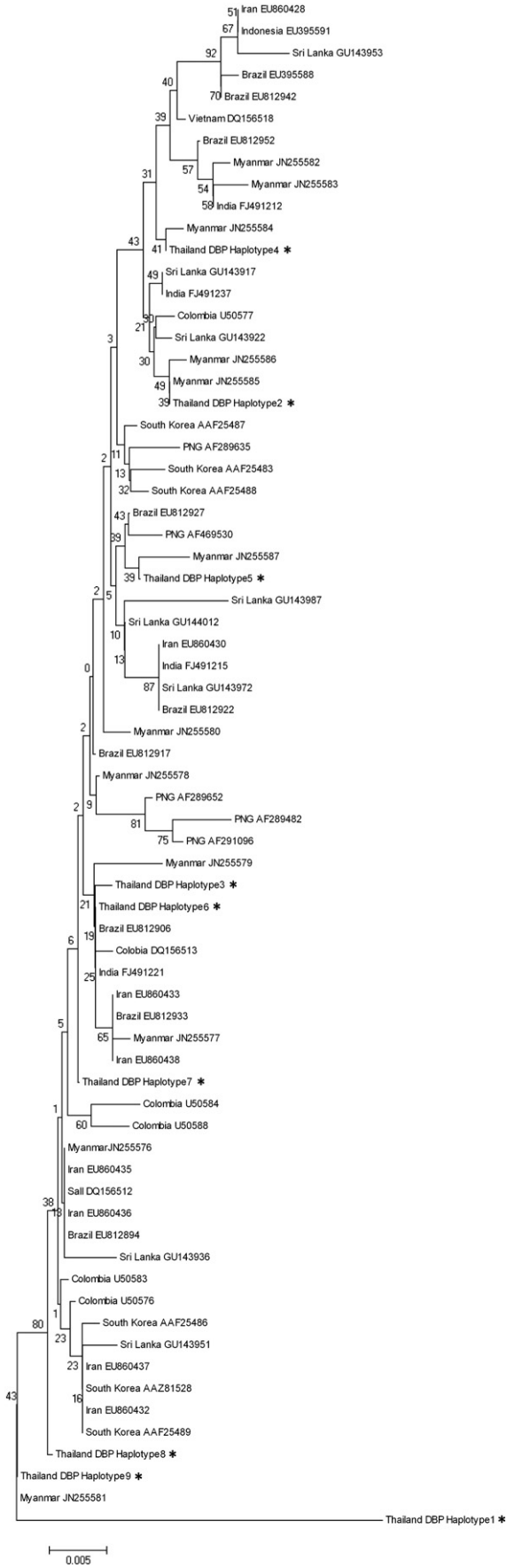


Fig. 1. The genetic polymorphism of PvDBPII among Thai isolates. (A) Polymorphic amino acid changes are listed for each Thai DBL haplotype relative to reference sequence Sal I (P22290.2). (B) Frequency of amino acid change at variant residues found in Thai DBL haplotypes.



3.3. Inhibitory function of anti-DBPII neutralizing antibodies against Thai DBPII variants

To determine if there are common epitopes associated with inhibition among Thai DBPII variants, the inhibitory function of anti-DBPII neutralizing antibodies against Thai DBPII haplotypes was assessed by in vitro COS7 assay of Thai DBPII-erythrocyte binding. The inhibitory efficiency of monoclonal antibodies 3C9, 2D10, 2C6 and 2H2 was measured against DBPII-DBL7.18-erythrocyte binding. First, the limiting dilution of each monoclonal antibody in the range of concentration to get 100% to 0% inhibition activity against PvDBPII7.18-erythrocyte binding was determined. The results showed inhibitory activity against DBPII-DBL7.18-erythrocyte binding in a dose dependent manner (Fig. 3). The 50% inhibitory concentrations (IC50) of 3C9, 2D10, 2C6 and 2H2 monoclonal antibodies were 0.21, 0.38, 2.61 and 0.64 μg/ml, respectively (Fig. 3A–D). We used the IC50 of each monoclonal antibody to evaluate the functional activity of anti-DBPII monoclonal antibody against the panel of Thai DBPII variants and homologous DBL7.18 expressed in the COS7 cell assay. The results revealed little variation in the inhibitory activity of monoclonal antibody 2H2 (Fig. 4D), suggesting that the epitope target of this anti-DBPII neutralizing antibody was conserved among DBL-TH variants. In contrast, there was a significant difference of inhibitory activity of monoclonal antibodies 3C9, 2D10 and 2C6 against the panel of Thai DBL variants ($P < 0.005$) (Fig. 4A–C). The results indicate that variant epitopes among Thai isolates were the target of most anti-DBPII neutralizing antibodies.

4. Discussion

Malaria is a major public health problem in Thailand. The surveillance data show that malaria transmission is found in nearly all areas of the country but is most prevalent along the border regions, especially the Thai–Myanmar border. Current public health surveys indicate vivax malaria prevalence has been on the rise in Thailand [21,22]. A significant portion of malaria cases in Thailand occur among temporary migrant workers from bordering countries presenting a major challenge to prevention and control of malaria in Thailand. The genetic polymorphism in the reticulocyte binding protein (RBP), merozoite surface protein 5 (MSP-5) and merozoite surface protein 3 α/β of *P. vivax* Thai isolates were analyzed in previous studies and showed high levels of genetic polymorphism [23–26]. However, the information on the nature and extent of population diversity as well as the antigenic character of malaria parasites in Thailand is still limited. In this study, genetic polymorphism and its potential relationship to antigenicity of Thai PvDBPII haplotypes were analyzed in order to support development of a strategy for *P. vivax* vaccine design in Thailand.

The 44 PvDBPII Thai isolates in this study were classified into 9 different haplotypes. Haplotype DBL-TH1 was identical to the PvDBPII sequence in a Myanmar isolate whereas the other 8 haplotypes were novel. A total of 12 polymorphic residues were identified which result in significant amino acid changes in PvDBPII Thai isolates. All variant residues of Thai PvDBPII have been previously reported in global vivax isolates [12,13,17,27]. The most prevalent polymorphic variants in Thai PvDBPII variants were L333F (78.9%), L424I (73.7%), W437R (73.4%) and I503K (63.2%) variants. Analysis of the L424I, W437R and I503K variants showed that the L424I–W437R, L424I–I503K and L424I–W437R–I503K combinations occurred with high frequencies of 73.7%, 57.9% and 57.2%, respectively. A previous study demonstrated that the W437R variant combined with the N417K or I503K variant can alter DBP antigenic character and can affect the efficiency of inhibitory antibodies against DBPII-erythrocyte binding [15]. Therefore,

Fig. 2. Phylogenetic analysis. The phylogenetic tree of the 9 Thai DBPII haplotypes was constructed with a neighbor-joining method using MEGA4 program. Numbers of the branches indicate bootstrap proportion (1000 replicates). The novel Thailand DBPII haplotypes are marked with asterisks.

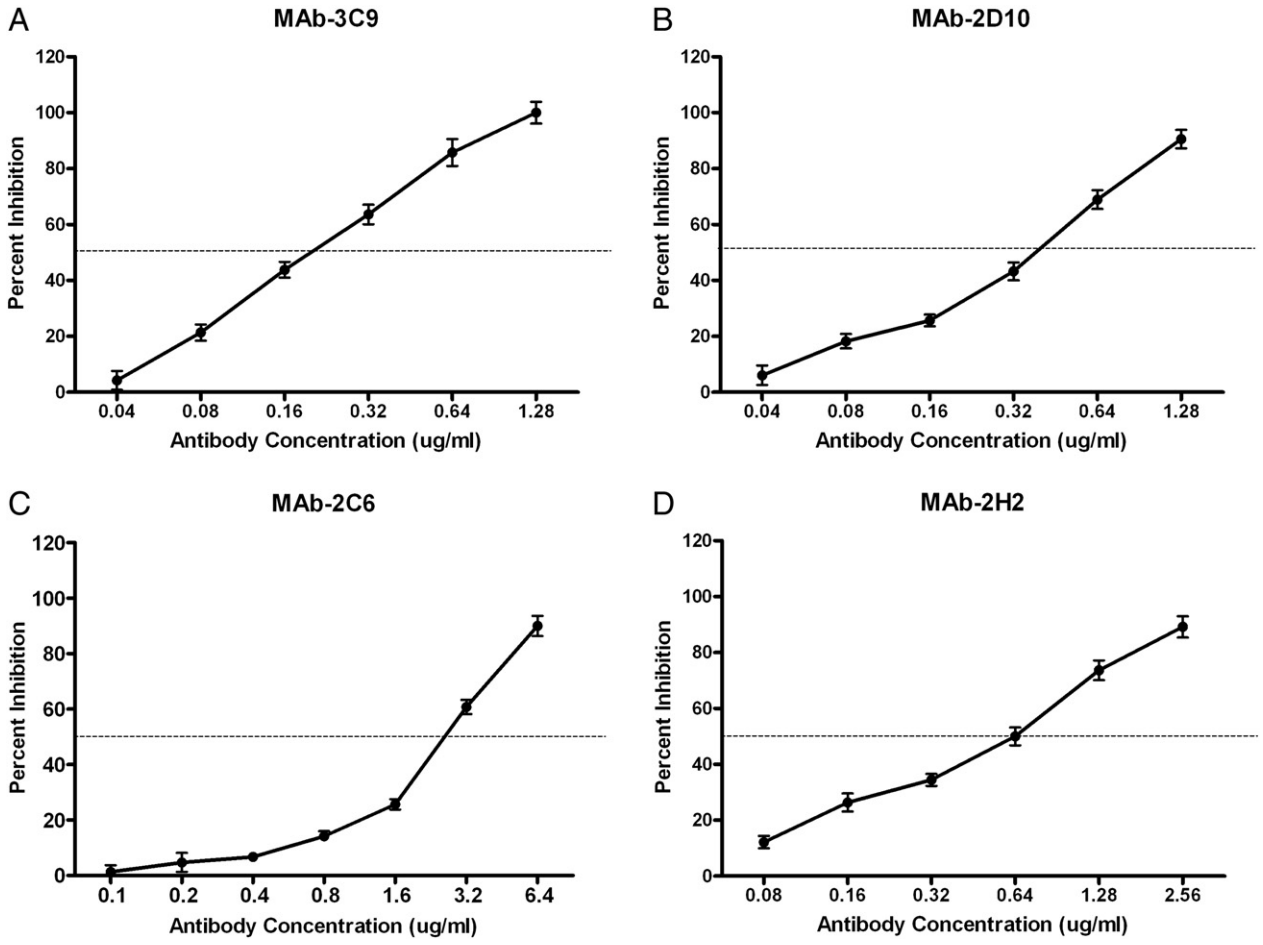


Fig. 3. Determination of 50% inhibitory concentration (IC50) of anti-DBPII monoclonal antibodies. Monoclonal antibodies 3C9, 2D10, 2C6 and 2H2 specific to PvDBPII 7.18 were tested for their inhibitory function against the homologous PvDBPII 7.18 haplotype. The transfected COS7 cells expressing PvDBPII 7.18 allele were incubated with monoclonal antibodies at different concentrations and with human erythrocytes. The number of rosettes was compared between wells of transfected cells incubated with monoclonal antibodies relative to wells without antibodies. Curves represent mean percent inhibition of two experiments tested in triplicate. Dotted line shows point on curve where IC50 of each antibody is achieved.

the combination of the L424I, W437R and I503K variants may also alter antigenicity and thus, the anti-DBPII response. Additional studies of naturally-acquired immunity against Thai DBPII protein antigens will be important for effective vaccine design.

Phylogenetic analysis revealed that Thai DBPII isolates had broad genetic diversity. A total of 9 Thai PvDBPII haplotypes were distributed among other geographic regions: Brazil, Papua New Guinea, Myanmar, Vietnam, Indonesia, Iran, Colombia, Sri Lanka, South Korea and India. The PvDBPII phylogeny showed that Thai PvDBPII isolates shared a common ancestor close to Myanmar isolates. The highest prevalence of L333F, L424I, W437R and I503K variants was common in Thai and Myanmar isolates [12]. However, there are 11 polymorphisms (I310L, F344S, R391H, K455I, K473R, C477G, R490K, D528G, V533M, K541T and A545V) found only in Myanmar isolates and not present in Thai isolates [12]. Taken together, these results indicate that immigration and/or travel are likely to influence the frequency of PvDBPII haplotypes between Thailand and Myanmar.

A significant challenge for DBPII vaccine development is its high level of polymorphism and the resulting strain-specific immunity. Previous studies have shown that individuals exposed to *P. vivax* parasites develop naturally acquired immunity [5,18,19]. However, most individuals develop strain specific immunity, with only a few individuals capable of producing broadly neutralizing anti-DBPII antibodies [28]. Vaccine design needs to identify neutralizing antibodies against conserved epitopes while avoiding presentation of immunogenic variant epitopes in a potential DBPII vaccine. Our objective was to evaluate the activity of broadly inhibitory anti-DBPII monoclonal antibodies against the diverse Thai DBPII variants identified in this study.

We analyzed the ability of anti-PvDBPII antibodies to inhibit Thai PvDBPII variant–erythrocyte binding by using an in vitro COS-7 erythrocyte-binding-inhibition assay. The concentration of monoclonal antibodies to achieve 50% inhibition against the homologous strain (IC50) was used to compare the inhibition efficiency against Thai PvDBPII variants. Monoclonal antibody 3C9 (IC50 = 0.21) had the strongest inhibition activity against homologous DBP7.18, followed by 2D10 (IC50 = 0.38), 2H2 (IC50 = 0.64) and 2C6 (IC50 = 2.61), respectively. A significant difference in inhibitory function against heterologous Thai variants was found in the 3C9, 2D10 and 2C6 monoclonal antibodies (Fig. 4A–C, $P < 0.005$), indicating that the these monoclonal antibodies may bind to different epitopes of Thai DBPII variants. Immune activity of 3C9, 2D10 and 2C6 monoclonal antibodies showed a trend toward variant-specific antibody with the individual Thai DBPII alleles. These results strongly support that the genetic polymorphisms of PvDBPII can alter antigenic character and thus may confer significant changes in their sensitivity to inhibitory function of anti-DBPII neutralizing antibodies as previously reported [8,15,19,28].

Immunization with PvDBP7.18 antigen showed a higher inhibitory response against Thai DBPII variants than against the homologous 7.18 haplotype. This data is supported by our observation that Mab 2C6 showed stronger inhibitory activity against DBPII-Sal I than against homologous DBPII7.18 (2C6; IC50 = 1.01 μg/ml in DBPII Sal I, IC50 = 2.61 μg/ml in DBPII7.18, data not shown). Our data is also in line with

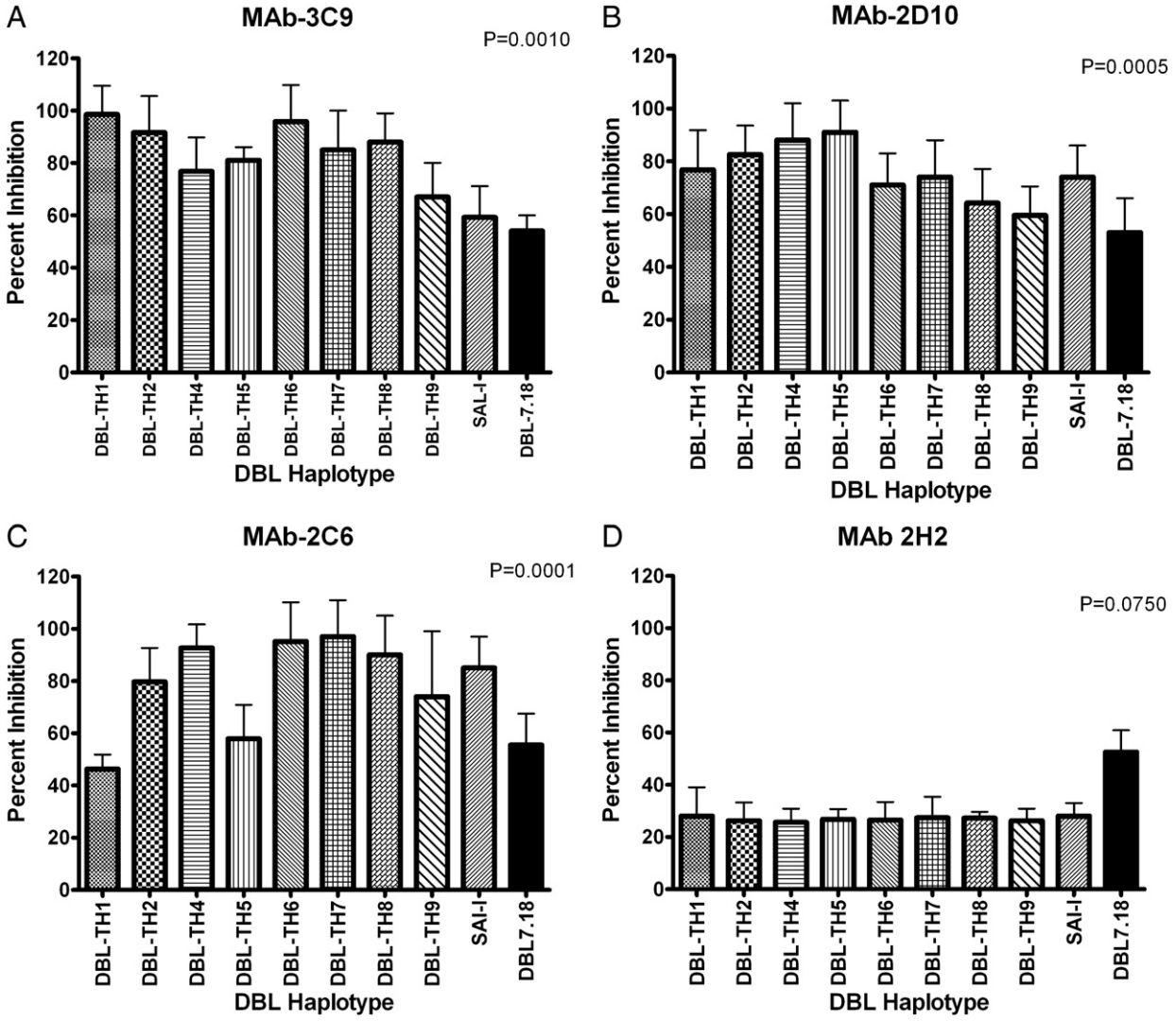


Fig. 4. Functional inhibition of anti-DBPII monoclonal antibodies against the panel of DBL-TH variants. The transfected COS7 cell expressing DBPII reference Sal I or 7.18 or DBL-TH variants were pre-incubated with monoclonal antibodies at concentrations set to the IC50 of the DBP7.18 haplotype antigen for inhibition of DBPII-erythrocyte binding. The charts show the mean inhibition of each DBL-TH variant. The variation in means across DBL-TH variants shows significant differences in the inhibitory responses of 3C9, 2D10 and 2C6 monoclonal antibodies. COS7 experiments for each monoclonal antibody were done in triplicate wells of each DBL variant and were repeated two times. Statistical significance was determined using one-way analysis of variance (ANOVA).

a previous study which showed that the alteration of variant residues on DBPII enhanced sensitivity to heterologous anti-DBPII antibody while other amino acid changes in the same residues increased refractoriness to antibody inhibition [15].

Interestingly, a number of DBPII neutralizing epitopes are shared among Thai DBPII variants. We have shown that a conserved epitope shared among Thai PvDBPII variants appears to be the target of the 2H2 anti-DBPII neutralizing monoclonal antibody (Fig. 4D). This data strongly supports that DBPII immunity has the potential to generate neutralizing antibodies against a range of variant haplotypes for broader strain recognition. The development of a broadly protective anti-DBPII vaccine to the dominant DBPII variants in field parasites will require induction of antibodies targeting epitopes that are conserved among variant DBPII alleles, such as those recognized by the 2H2 monoclonal antibody indicated in this study.

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